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A Live-Attenuated Chimeric Porcine Circovirus Type 2 (PCV2) Vaccine Is Transmitted to Contact Pigs but Is Not Upregulated by Concurrent Infection with Porcine Parvovirus (PPV) and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Is Efficacious in a PCV2b-PRRSV-PPV Challenge Model[▽]

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The live chimeric porcine circovirus type 2 (PCV2) vaccine with the capsid gene of the emerging subtype 2b cloned in the genomic backbone of the nonpathogenic PCV1 is attenuated *in vivo* and induces protective immunity against PCV2. To further determine the safety and efficacy of this experimental vaccine, we tested for evidence of pig-to-pig transmission by commingling nonvaccinated and vaccinated pigs, determined potential upregulation by simultaneous vaccination and infection with porcine parvovirus (PPV) and porcine reproductive and respiratory syndrome virus (PRRSV), and determined vaccine efficacy by challenging pigs 4 weeks after vaccination with PCV2b, PRRSV, and PPV. Forty-six 21-day-old, PCV2-naïve pigs were randomly assigned to one of six groups. Twenty-nine of 46 pigs were challenged with PCV2b, PRRSV, and PPV at day 28, 8/46 remained nonvaccinated and nonchallenged and served as negative controls, and 9/46 remained nonchallenged and served as vaccination controls. All animals were necropsied at day 49. PCV1-PCV2 viremia was detected in nonvaccinated contact pigs commingled with vaccinated pigs, indicating pig-to-pig transmission; however, PCV1-PCV2 DNA levels remained low in all vaccinated and contact pigs regardless of concurrent infection. Finally, vaccination 28 days before challenge resulted in significantly ($P < 0.05$) decreased amounts of PCV2 in tissues and sera and significantly ($P < 0.05$) reduced macroscopic and microscopic lesions. The results of this study indicate that the experimental live-attenuated chimeric PCV2 vaccine, although transmissible to contact pigs, remains attenuated in pigs concurrently infected with PRRSV and PPV and induces protective immunity against PCV2b when it is administered 28 days before PCV2 exposure.

Porcine circoviruses (PCVs) comprise a group of small, nonenveloped, single-stranded circular DNA viruses (44) which can be divided into two major genotypes: nonpathogenic PCV type 1 (PCV1) and pathogenic PCV type 2 (PCV2) (1). Moreover, PCV2 can be subdivided into two major subtypes, commonly referred to as PCV2a and PCV2b (6, 40), from which the latter subtype (PCV2b) emerged recently in the United States and Canada. Porcine circoviruses contain two major open reading frames (ORFs) oriented in opposite directions which encode proteins associated with replication (ORF1) and the capsid (ORF2) (21, 25).

Commercial PCV2 vaccines for use in growing pigs and breeding-age animals became available in North America in 2006, and at least four commercial vaccines are now available. All products available to date are inactivated or subunit vaccines based on the PCV2a subtype, even though the currently dominant strain circulating in the field is PCV2b. Studies have shown that PCV2a and PCV2b are cross-protective (12, 34). Under experimental (12, 29, 31, 32) and field (8, 17, 19) conditions, the current inactivated or subunit PCV2 vaccines have been shown to be extremely effective in reducing PCV2 viremia, PCV2-associated microscopic lesions, and PCV2-associated morbidity and mortality (12, 29, 31, 32). In addition, vaccinated pigs were shown to have improved average daily gain, increased percentage of lean meat yield, improved feed conversion, and decreased back fat depth, and typically the medication costs in vaccinated herds were reduced (8, 17, 19). Several of the commercially available killed PCV2 vaccines require 1 or 2 doses of intramuscular administration (30).

An experimental live chimeric PCV1-PCV2a (PCV1-2a) vaccine with the capsid gene of PCV2a cloned in the backbone of the nonpathogenic PCV1 has been developed and shown to be nonpathogenic in the growing-pig model (11). An inactivated version of the live vaccine, Suvaxyn PCV2 (Fort Dodge Animal Health, Inc.), has previously been licensed and was commercially introduced to the North American pig population in 2006 (30). Both the inactivated and the live-attenuated PCV2 vaccines were demonstrated to be very effective and

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induced protective immunity in the singular PCV2-challenge model (10, 31, 33, 43). It has been shown that the live chimeric PCV1-2a vaccine virus is genetically stable when it is serially passaged in cell culture as well as in pigs (14). Interestingly, in 2008 a chimeric PCV1-2a was isolated from cases of acute outbreaks of porcine reproductive and respiratory syndrome (PRRS) in Canada (13). The authors speculated that the identified PCV1-2 may have originated from the chimeric killed vaccine widely used in the affected areas. Alternatively, formation of PCV1-2a may have been due to a natural recombination event between PCV1 and PCV2 (13).

Globally, indications are that PCV2b is by far the most common strain associated with PCV2-associated diseases (PCVADs); however, all current commercial vaccines, including the experimental live PCV2 vaccine described above, are based on the PCV2a subtype. Thus, in an effort to continuously improve existing products, it seems important to evaluate a PCV2 vaccine that is based upon the predominant 2b subtype.

Recently, we succeeded in developing a live-attenuated chimeric PCV1-2b vaccine and have shown that the novel PCV1-2b vaccine virus is attenuated and induces homologous and heterologous immunity against both PCV2b and PCV2a subtypes (4), making it a promising candidate as a live-attenuated vaccine against both PCV2b and PCV2a. However, before a live-attenuated vaccine can be introduced to the pig market, it is of particular importance to further determine its safety. It has been well documented that concurrent infections of PCV2 with other swine pathogens such as PRRS virus (PRRSV) (3, 16, 39), porcine parvovirus (PPV) (2, 20), swine torque teno virus (TTV) (7), or *Mycoplasma hyopneumoniae* (35) enhance PCV2 replication and PCVAD. The consequences of concurrent infections with the chimeric PCV1-2b vaccine virus with other pathogens are unknown. Therefore, it is of paramount importance to make sure that the live PCV2 chimeric vaccine virus is safe in vaccinated pigs coinfected with other common swine pathogens.

The objectives of this study were to determine the (i) possible horizontal spread (objective 1), (ii) safety (objective 2), and (iii) efficacy (objective 3) of a chimeric PCV1-2b vaccine in a PCV2-PRRSV-PPV triple-challenge model. For objective 1, nonvaccinated contact pigs were commingled with vaccinated pigs to determine if the live chimeric PCV2 vaccine was transmitted to nonvaccinated animals. For objective 2, pigs were vaccinated with the PCV1-2b vaccine and on the same day infected with PRRSV and PPV, two pathogens that are endemic in the pig population and that have both been shown to enhance PCV2 replication and disease. For objective 3, pigs were vaccinated with the PCV1-2 vaccine and challenged with PCV2b, PPV, and PRRSV at 28 days after vaccination, and protection against PCV2 infection was measured by determining the presence and degree of PCV2 viremia and PCV2-associated lesions.

MATERIALS AND METHODS

Chimeric PCV1-2b vaccine virus. The chimeric PCV1-2b vaccine virus was created by cloning the capsid gene of the new PCV2b subtype into the genomic backbone of the nonpathogenic PCV1, and it was previously demonstrated to be attenuated and to induce protective and cross-protective immunity against PCV2a and PCV2b (4). A subclone of the PK-15 cell line that is free of PCV1 contamination was used for the generation of PCV1-2b vaccine virus stocks as described previously (4, 9, 10). The 50% tissue culture infective dose ($TCID_{50}$) per ml was calculated according to the method of Reed and Muench. The PCV1-2b chimeric vaccine virus stock had an infectious titer of 10^4 $TCID_{50}$s per ml. Each pig received 4.5 ml of the vaccine virus stock (2.5 ml intranasally and 2 ml intramuscularly into the right neck area). The reason for using intranasal and intramuscular routes in this small-scale study was to ensure equal exposure of all pigs to the live chimeric PCV2 vaccine and to activate both the mucosal and systemic immune responses.

TABLE 1. Experimental design

Group	No. of pigs	PCV1-2b vaccination		PCV2b-PRRSV-PPV challenge, trial day 28 (age 49 days)
		Trial day 0 (age 21 days)	Trial day 28 (age 49 days)	
Negative controls	8	−	−	−
Positive controls	8	−	−	+
Vac-0	9	+	−	−
Vac-0–PCV2	9	+	−	+
Contact-PCV2[a]	3	−	−	+
Vac-28–PCV2	9	−	+	+

[a] Contact-PCV2 pigs were moved into the pens with the Vac-0–PCV2 group 3 days after PCV1-2b vaccination.

Animals, housing, and experimental design. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. Forty-six pigs were obtained from a commercial swine herd free of PCV2, PRRSV, swine influenza virus, and PPV as determined by routine serology. At arrival, the 46 pigs were randomly divided (off the truck by gate cut) into six treatment groups and five rooms as outlined in Table 1. Each room contained three raised wire decks 2.5 by 3.6 m equipped with one nipple drinker and one self-feeder. At day 0 (when the pigs were approximately 21 days of age), pigs in group Vac-0 and group Vac-0–PCV2 were each vaccinated with approximately 4.5×10^4 $TCID_{50}$s of the PCV1-2b vaccine by intranasal (2.5 ml) and intramuscular (2 ml) injection (20-gauge needle, right neck area). At day 3, the three nonvaccinated pigs in the contact-PCV2 group were moved into the same room as the group Vac-0–PCV2 pigs and commingled at a ratio of three vaccinated (Vac-0–PCV2) pigs to one nonvaccinated contact-PCV2 pig in each pen. At day 28, group Vac-28–PCV2 pigs were each inoculated with the PCV1-2b vaccine intranasally and intramuscularly as described above. All pigs in the positive-control, Vac-0–PCV2, contact-PCV2, and Vac-28–PCV2 groups were challenged with PCV2b, PRRSV, and PPV at trial day 28. Throughout the experiment, the pigs were weighed and bled weekly. After challenge, the pigs were monitored daily for clinical signs of disease. At trial day 49 (21 days after PCV2-PRRSV-PPV challenge), all pigs were necropsied by a pathologist blinded to treatment group. Various tissues were collected during the necropsy and evaluated in a blinded fashion for severity and characteristics of macroscopic and microscopic lesions as described previously (35).

PCV2b-PRRSV-PPV challenge. PCV2b isolate NC-16845 was used for the challenge and was propagated on PK-15 cells as previously described (34). The PCV2b challenge virus stock had an infectious titer of $10^{4.0}$ $TCID_{50}$s per ml. PRRSV isolate ATCC VR2385 was propagated on MARC-145 cells. The 7th passage of the virus, with a titer of 1×10^5 to 1×10^6 focus-forming units/ml, which corresponds to approximately 1×10^5 $TCID_{50}$s/ml (18), was used as the challenge stock. PPV strain NADL-8 was isolated from fetal porcine kidney cells from a naturally infected pig in 1977 in Perry, IA (22). In this study, the challenge virus stock was PPV from passage 4 in pigs (10% tissue homogenate suspension containing lungs of aborted fetuses in minimal essential medium) from the year 1982 and was used at an approximate titer of $10^{4.9}$ $TCID_{50}$s. Each pig in the challenged groups (Table 1) received 2 ml of the PCV2b stock intramuscularly into the right neck. In addition, each pig also received 2 ml PCV2b, 3 ml PRRSV, and 1 ml PPV intranasally by slowly dripping each inoculum into both nostrils. Successful challenge using this model was demonstrated by seroconversion, detected by enzyme-linked immunosorbent assay (ELISA), and viremia, detected by real-time PCR in the unvaccinated positive-control group.

Serology. Blood samples were collected upon arrival of the pigs and weekly thereafter until necropsy at trial day 49. All serum samples were tested by an ORF2-based anti-PCV2 IgG ELISA (24). Samples were considered positive if the calculated sample-to-positive (S/P) ratio was 0.2 or greater (24). All serum samples collected on trial days 28, 35, 42, and 49 were also tested for the presence of specific antibodies to PRRSV with a commercial PRRSV ELISA (HerdChek

PRRS 2XR antibody ELISA; IDEXX Laboratories, Inc. Westbrook, MA). A hemagglutination inhibition assay for detection of PPV-specific antibodies was performed on all serum samples on trial days 28 (challenge) and 49 (necropsy) as previously described (23).

Clinical evaluation. Following PCV2 inoculation, the pigs were monitored daily for clinical signs, including sneezing, lethargy, and coughing. All pigs were weighed once per week.

Quantification of PCV2 and PCV1-2b DNA loads. DNA was extracted from serum samples collected on trial day 0 and weekly thereafter until termination of the study using a QIAamp DNA minikit (Qiagen, Valencia, CA). DNA extracts were used for quantification of PCV2 genomic DNA copy numbers by real-time PCR as described previously (36). All DNA extracts from trial days 0 to 49 were used for quantification of the vaccine virus PCV1-2b genomic DNA copy numbers by a real-time PCR assay designed to differentiate PCV1-2 chimeric DNA from both PCV2 and PCV1 DNA. A forward primer (5′-TGACAGTATATCC GAAGGTGCG-3′), a reverse primer (5′-GCCGAAGTGCGCTGGTAATA-3′), and a probe (5′-CAL Fluor Orange 560-CGCACTTCTTTCACTTTTATA GGATG-BHQ-3′) were designed in the region spanning PCV2 ORF2 (GenBank accession no. AF264042) and PCV1 ORF1 (GenBank accession no. U49186) using the primer-probe test tool of Primer Express software, version 3.0 (Applied Biosystems, Foster City, CA). The presence of sequence dissimilarity in the regions allowed PCV1-2b chimeric DNA to be distinguished from both PCV2 and PCV1 DNA. The real-time PCR mixture consisted of a total volume of 25 μl containing 12.5 μl of TaqMan universal PCR master mix (Applied Biosystems), 1 μl of each forward and reverse primer (final concentration, 400 nM), 0.5 μl TaqMan probe (final concentration, 200 nM), 7.5 μl nuclease-free water, and 2.5 μl extracted DNA. The reactions were carried out in a 7500 Fast real-time PCR system (Applied Biosystems). The PCR cycling parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 55°C, and 1 min at 60°C. Samples were considered negative when there was no observed threshold cycle (C_T) value during the 40 amplification cycles. Five progressive 1:10 dilutions of a known copy number (8.13×10^9 to 8.13×10^4 copies/ml) of the PCV1-2b chimeric DNA clone in the pSK vector were used to generate the standard curve. The specificity of the primers and probe was determined by using 2.5 μl PCV1, PCV2a, and PCV2b DNA extracted from virus preparations and animals infected with PCV2a or PCV2b as described above. No cross-reactions were observed in the real-time PCR (data not shown).

PRRSV RNA quantification. Total RNAs from the serum samples obtained on trial days 35, 42, and 49 were isolated using a QIAamp viral RNA minikit (Qiagen) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (Q-RT-PCR) was performed using TaqMan PRRSV reagents and controls (Applied Biosystems) in a 25-μl volume with 12.5 μl 2× multiplex RT-PCR buffer, 2.5 μl 10× PRRSV primer-probe mix, 1.25 μl 20× multiplex enzyme mix, 0.75 μl nuclease-free water, and 8 μl of PRRSV RNA standard or extracted RNA. The reaction mixture was incubated at 45°C for 10 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 70 s.

PPV DNA quantification. DNA was extracted from the serum samples at trial day 49 using a QIAamp DNA blood minikit (Qiagen) and tested by a quantitative PPV real-time PCR. A forward primer (5′-CAGAATCAGCAACCTCACC A-3′), a reverse primer (5′-GCTGCTGGTGTGTATGGAAG-3′), and a probe (5′–6-carboxyfluorescein–TGCAAGCTTAATGGTCGCACTAGACA–black hole quencher–3′) were designed to amplify the VP2 gene of PPV. The reaction was performed in a 25-μl mixture with 12.5 μl 2× PCR master mix (Applied Biosystems), 1 μl of each forward and reverse primer (final concentration, 500 nM), 0.5 μl TaqMan probe (final concentration, 200 nM), 7.5 μl nuclease-free water, and 2.5 μl extracted DNA. The amplification was performed at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Serial dilutions of a recombinant vector PCR2.1 (Invitrogen, Carlsbad, CA) containing the PPV VP2 gene were used to obtain a standard curve.

Necropsy. All pigs were euthanized by intravenous pentobarbital sodium (Fatal-Plus; Vortech Pharmaceutical, LTD, Dearborn, MI) overdose and necropsied on trial day 49. The total amount of macroscopic lung lesions (range, 0 to 100%) was estimated and scored as previously described (15). Additionally, the sizes of lymph nodes (tracheobronchiolar, mediastinal, and superficial inguinal), ranging from 0 (normal) to 3 (four times the normal size), were estimated and recorded (27). Sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric, and external iliac), tonsil, thymus, ileum, kidney, colon, spleen, and liver were collected at necropsy, fixed in 10% neutral-buffered formalin, and routinely processed for histological examination.

Histopathology. Microscopic lesions were evaluated by two veterinary pathologists (T.O., P.G.H.) blinded to the treatment group. Sections were scored for the presence and severity of interstitial pneumonia, with scores ranging from 0 (normal) to 6 (severe diffuse) (15). Sections of heart, liver, kidney, ileum, and colon were evaluated for the presence of lymphohistiocytic inflammation and scored from 0 (none) to 3 (severe). Lymphoid tissues, including lymph nodes (tracheobronchiolar, mesenteric, mediastinal, superficial inguinal, and external iliac), tonsil, and spleen were evaluated for the presence of lymphoid depletion, with scores ranging from 0 (normal) to 3 (severe), and histiocytic inflammation and replacement of follicles, with scores ranging from 0 (normal) to 3 (severe) (35). The overall microscopic lymphoid lesion score, which consists of the scores for lymphoid depletion, histiocytic inflammation, and the presence of PCV2 antigen in lymphoid tissues, was calculated as previously described and ranged from 0 (normal) to 9 (severe) (35). Briefly, the scores (lesions and PCV2 immunohistochemistry [IHC]) of seven lymphoid tissues ([lymph node pool] × 4, tracheobronchial lymph node, spleen, and tonsil) were added up and divided by 7. The lymph node pool consisted of superficial inguinal, external iliac, mediastinal, and mesenteric lymph nodes. The mean treatment group lymphoid score was calculated and compared between groups. Pigs were grouped into four categories on the basis of overall microscopic lymphoid lesion scores: 0 (normal), 1 to 3 (mild), 4 to 6 (moderate), and 7 to 9 (severe) (35).

IHC. Detection of PCV2-specific antigen was performed on selected formalin-fixed and paraffin-embedded sections of lymph nodes (superficial inguinal, mediastinal, tracheobronchial, external iliac, and mesenteric), tonsil, spleen, and thymus using a rabbit polyclonal antiserum specific for PCV2 capsid protein (42). PCV2 antigen scoring was done by a veterinary pathologist (T.O.) blinded to treatment. Scores ranged from 0 (no signal) to 3 (more than 50% of the lymphoid follicles contain cells with PCV2 antigen staining) (35).

Statistical analysis. Summary statistics were calculated to assess the overall quality of the data. Analysis of variance (ANOVA) was used for cross-sectional assessment of the average daily weight gain and repeated continuous measures. The significance level was set for a P value of <0.05, followed by pairwise testing using the Tukey-Kramer adjustments to identify the groups that were different. Nonrepeated measures of necropsy and histopathology data were assessed using nonparametric Kruskal-Wallis ANOVA. If a nonparametric ANOVA test was significant ($P < 0.05$), then Wilcoxon tests were used to assess the differences of pairs of groups. Differences in incidence were evaluated by using Fisher's exact test. Statistical analysis was performed using JMP software (version 8.0.0; SAS Institute Inc., Cary, NC).

RESULTS

Clinical presentation. Clinical disease was not observed in any of the pigs after PCV1-2b vaccination, and none of the pigs had visible reactions (redness, swelling, higher temperature) at the vaccination site. After PCV2, PRRSV, and PPV challenge at trial day 28, pigs in the positive-control, Vac-0–PCV2, contact-PCV2, and Vac-28–PCV2 groups developed mild respiratory disease characterized by sneezing and occasional clear nasal discharge. The average daily weight gain was not different among groups before or after challenge (data not shown). However, individual pigs in the positive-control group and the Vac-28–PCV2 group had a decreased weight gain after challenge. Between trial days 35 and 42, 1/8 positive-control pigs had decreased weight gain (0.3 lb), became lethargic, and died at trial day 45 (18 days postchallenge). Between trial days 42 and 49, 3/9 Vac-28–PCV2 pigs lost weight (−0.4, −0.6, and −0.6 lb, respectively).

Macroscopic lesions. At necropsy, the majority of the challenged pigs had macroscopic lung lesions characterized by multifocal-to-diffuse, mottled-tan areas of consolidation and diffuse failure of the lungs to collapse. The incidence of pigs with lung lesions was 0/8 negative controls, 7/8 unvaccinated positive controls, 2/9 Vac-0 pigs, 7/9 Vac-0–PCV2 pigs, 3/3 contact-PCV2 pigs, and 9/9 Vac-28–PCV2 pigs. The mean group lung lesion scores are summarized in Table 2. Unvaccinated positive controls and Vac-28–PCV2 pigs had significantly ($P < 0.05$) higher mean gross lung lesion scores than all other groups. The majority of the pigs also had evidence of lymph node enlargement up to 3 times the normal size (2/8 negative controls, 7/8

TABLE 2. Macroscopic and microscopic lung lesions and prevalence of PCV2 and PRRSV antigen in lung tissues[a]

Group	No. of pigs	Lung lesion score		Prevalence of antigen (no. of pigs with antigen/total no. in group)	
		Macroscopic	Microscopic	PCV2	PRRSV
Negative controls	8	0.0 ± 0.0^{A}	$1.4 \pm 0.2^{A,B}$	0/8	0/8
Positive controls	8	46.9 ± 8.1^{B}	4.1 ± 0.3^{C}	2/8	3/8
Vac-0	9	1.8 ± 1.4^{A}	0.6 ± 0.2^{B}	0/9	0/9
Vac-0–PCV2	9	18.2 ± 4.9^{A}	2.6 ± 0.4^{A}	0/9	1/9
Contact-PCV2	3	13.3 ± 6.2^{A}	$2.7 \pm 0.3^{A,C}$	0/3	1/3
Vac-28–PCV2	9	47.2 ± 8.4^{B}	3.8 ± 0.2^{C}	2/9	2/9

[a] Data represent group means ± standard errors for macroscopic lung lesions (with a range of 0 to 100% of the lung surface affected by lesions) and microscopic lung lesions (with a range of 0 [normal] to 6 [severe diffuse interstitial pneumonia) and prevalence of PCV2 and PRRSV antigen in lung tissues determined by immunohistochemical stains at the time of necropsy (trial day 49, 21 days after PCV2b-PRRSV-PPV challenge). Different superscripts within columns (A, B, C) indicate significant ($P < 0.05$) differences between groups.

unvaccinated positive controls, 1/9 Vac-0 pigs, 8/9 Vac-0–PCV2 pigs, 3/3 contact-PCV2 pigs, and 9/9 Vac-28—PCV2 pigs).

Serological response. (i) Anti-PCV2-IgG antibody response. The negative-control piglets remained negative for anti-PCV2 antibodies through the end of the study (Table 3; Fig. 1), whereas the positive-control piglets began to seroconvert at 14 days postchallenge (trial day 42). Pigs vaccinated with PCV1-2b on trial day 0 (Vac-0 and Vac-0–PCV2) seroconverted between trial days 14 and 28. The nonvaccinated contact-PCV2 pigs seroconverted between trial days 35 and 42 after PCV2 challenge (Table 3).

(ii) Anti-PRRSV antibody response. All pigs were negative for anti-PRRSV antibodies prior to initiation of the study and at trial day 35. Negative controls and Vac-0 pigs remained PRRSV seronegative for the remainder of the study. At trial day 42, 3/7 unvaccinated positive controls, 9/9 Vac-0–PCV2 pigs, 3/3 contact-PCV2 pigs, and 7/9 Vac-28–PCV2 pigs were positive for anti-PRRSV antibodies. At trial day 49, all pigs inoculated with PRRSV had seroconverted to PRRSV, except for 1/9 Vac-28–PCV2 pigs (data not shown).

(iii) Anti-PPV antibody response. All pigs were negative for anti-PPV antibodies prior to and at trial day 28. At trial day 42, 0/8 negative controls, 7/7 unvaccinated positive controls, 9/9 Vac-0–PCV2 pigs, 2/3 contact-PCV2 pigs, and 9/9 Vac-28–PCV2 pigs were positive for anti-PPV antibodies (data not shown).

Prevalence and PCV2 DNA load in serum. The group mean $\log_{10}$ numbers of PCV2 DNA genomic copies per ml of serum are summarized in Table 3. PCV2 DNA was not detected in any pig at the day of challenge (trial day 28) or in pigs in the negative-control group at any time point. Figure 2 shows a comparison of the PCV2 DNA load in sera of the pigs challenged with PCV2 (unvaccinated positive controls, Vac-0–PCV2, contact-PCV2, Vac-28–PCV2). While the PCV2 DNA load in serum was not different between the unvaccinated positive controls and the Vac-28–PCV2 pigs, it was significantly ($P < 0.05$) higher in the Vac-28–PCV2 pigs than in the Vac-0–PCV2 and contact-PCV2 groups (Fig. 2).

TABLE 3. Prevalence of PCV1-2b DNA, PCV2b DNA, and anti-PCV2 IgG antibodies and group mean $\log_{10}$ numbers of PCV2 DNA genomic copies/ml serum in pigs in the different groups on different trial days[a]

Group	DNA or AB	Result on trial day:							
		0	7	14	21	28	35	42	49
Negative controls	PCV1-2 DNA	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
	PCV2 DNA	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
	PCV2 AB	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
Positive controls	PCV1-2 DNA	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)
	PCV2 DNA	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	**8/8 (5.80)**	**8/8 (6.78)**	**7/7 (6.63)**
	PCV2 AB	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)	**7/8 (0.276)**	**7/7 (0.691)**
Vac-0	PCV1-2 DNA	0/9 (0.0)	**1/9 (2.91)**	**5/9 (3.09)**	**8/9 (4.01)**	**5/9 (3.66)**	**2/9 (3.24)**	**1/9 (3.45)**	**1/9 (2.88)**
	PCV2 DNA	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)
	PCV2 AB	0/9 (0.0)	0/9 (0.0)	**3/9 (0.253)**	**6/9 (0.324)**	**9/9 (0.399)**	**9/9 (0.432)**	**9/9 (0.541)**	**9/9 (0.580)**
Vac-0–PCV2	PCV1-2 DNA	0/9 (0.0)	**2/9 (3.56)**	**6/9 (3.30)**	**6/9 (3.65)**	**5/9 (3.29)**	**1/9 (3.73)**	**1/9 (3.50)**	**1/9 (2.96)**
	PCV2 DNA	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	**1/9 (3.05)**	**1/9 (3.22)**	**1/9 (2.90)**
	PCV2 AB	0/9 (0.0)	0/9 (0.0)	**1/9 (0.233)**	**6/9 (0.324)**	**9/9 (0.401)**	**9/9 (0.454)**	**9/9 (0.592)**	**9/9 (0.638)**
Contact-PCV2	PCV1-2 DNA	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	**2/3 (3.40)**	**3/3 (3.50)**	**2/3 (3.54)**	**1/3 (2.97)**
	PCV2 DNA	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	**3/3 (3.79)**	**2/3 (3.55)**	**2/3 (3.20)**
	PCV2 AB	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	**2/3 (0.245)**	**3/3 (0.457)**	**3/3 (0.503)**
Vac-28–PCV2	PCV1-2 DNA	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	**6/9 (3.30)**	**6/9 (4.02)**	**2/9 (3.64)**
	PCV2 DNA	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	**9/9 (6.39)**	**9/9 (6.59)**	**9/9 (5.61)**
	PCV2 AB	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	**5/9 (0.0)**	**9/9 (0.502)**

[a] Prevalence of PCV1-2b DNA, PCV2b DNA, and anti-PCV2 IgG antibodies (AB) in pigs in the different groups at trial days 0, 7, 14, 21, 28 (the day of PCV2b-PRRSV-PPV challenge), 35, 42, and 49 (the day of necropsy). Data are presented as number of positive animals/total number of animals (log-transformed mean genomic copy numbers/ml serum for PCR-positive animals or group mean S/P ratio for ELISA-positive animals). An S/P ratio equal to or greater than 0.2 was considered positive. Positive samples are indicated in boldface.

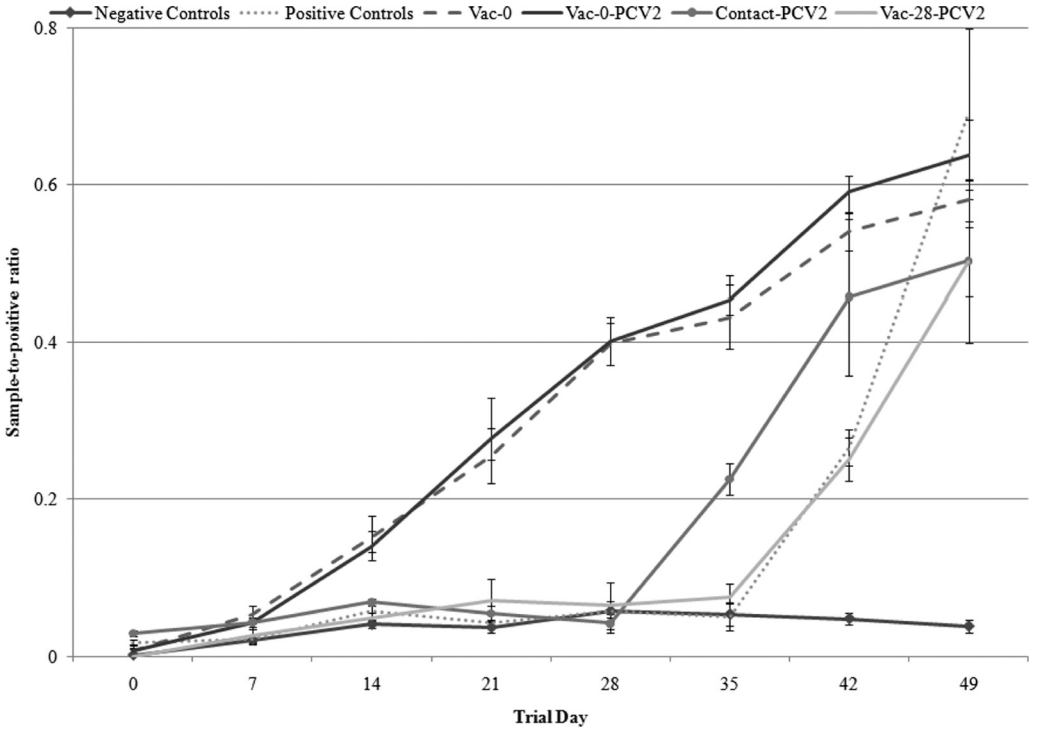

FIG. 1. Group mean sample-to-positive ratios and standard errors for anti-PCV2 IgG antibody response. Pigs in groups Vac-0 and Vac-0–PCV2 were PCV1-2 vaccinated at trial day 0. Pigs in groups Vac-28–PCV2 were PCV1-2 vaccinated at trial day 28. Contact-PCV2 pigs were nonvaccinated but commingled with Vac-0–PCV2 pigs. PCV2-PRRSV-PPV challenge was done at trial day 28. A sample-to-positive ratio equal to or greater than 0.2 was considered positive.

Prevalence and amount of PCV1-2 vaccine virus DNA in serum. The PCV1-2 chimeric vaccine virus DNA was detected in pigs 7 days after vaccination (Table 3). The amount of PCV1-2 DNA was not different among treatment groups, regardless of coinfection status. Two of three contact-PCV2 pigs became PCR positive for PCV1-2 at trial day 28, and by trial day 35, all three pigs in this group were positive for serum PCV1-2 DNA (Table 3).

Prevalence and amount of PRRSV RNA and PPV DNA load in serum. At trial day 49, PRRSV RNA was detected in 0/8 negative controls ($\log_{10}$ group mean ± standard error [SE], 0.0 ± 0.0), 6/7 unvaccinated positive controls (3.9 ± 0.9), 0/9 Vac-0 pigs (0.0 ± 0.0), 9/9 Vac-0–PCV2 pigs (4.6 ± 0.3), 3/3 contact-PCV2 pigs (3.8 ± 0.6), and 9/9 Vac-28–PCV2 pigs (4.8 ± 0.5). There was no significant difference in the amount of PRRSV RNA among groups. At trial day 49, PPV DNA was detected in 0/8 negative controls ($\log_{10}$ group mean ± SE, 0.0 ± 0.0), 6/7 unvaccinated positive controls (3.1 ± 0.5), 0/9 Vac-0 pigs (0.0 ± 0.0), 6/9 Vac-0–PCV2 pigs (2.1 ± 0.6), 2/3 contact-PCV2 pigs (2.6 ± 1.4), and 4/9 Vac-28–PCV2 pigs (1.2 ± 0.5). The positive-control pigs had significantly ($P < 0.05$) higher serum PPV DNA levels than Vac-28–PCV2 pigs.

Microscopic lesions and detection of intralesional PCV2 and PRRSV antigen. Microscopic lesions in lung tissues were characterized by multifocal-to-diffuse, mild-to-moderate bronchointerstitial pneumonia with type 2 pneumocyte hyperplasia and hypertrophy, thickening of alveolar septa with macrophages and neutrophils, and mild peribronchiolar fibrous hyperplasia. The group mean scores of interstitial pneumonia are summarized in Table 2. Positive-control pigs and Vac-28–PCV2 pigs had significantly ($P < 0.05$) more severe lung lesions than pigs in the other groups except contact-PCV2 pigs. PRRSV antigen was detected in individual pigs in all PRRSV-inoculated groups (Table 2). The presence of PCV2 antigen in lung tissues was limited to positive-control pigs and Vac-28–PCV2 pigs (Table 2). The group overall lymphoid lesion scores are summarized in Table 4. Three pigs (1/8 unvaccinated positive controls and 2/9 Vac-28–PCV2 pigs) were identified as having severe diffuse PCV2-associated microscopic lesions consistent with systemic PCVAD. PCV2 antigen was identified in individual pigs in the positive-control, Vac-0, contact-PCV2, and Vac-28–PCV2 pigs, with a higher prevalence in the unvaccinated positive controls and Vac-28–PCV2 pigs than all other groups (Table 4).

DISCUSSION

It is estimated that up to 99% of the market-age pigs in the U.S. swine population have been vaccinated against PCV2 (Edgar Diaz, Boehringer Ingelheim Vetmedica Inc., personal communication). With the widespread use of PCV2 vaccination, cases of suspected vaccine failure are emerging, and investigations are in progress to determine the reason(s). Reasons for vaccine failure can include errors in compliance with vaccine administration protocols, such as administering the wrong dose, using the wrong route of injection, or using heterologous vaccine strains with limited cross-protection. Vaccination at a time when pigs are going through infection with

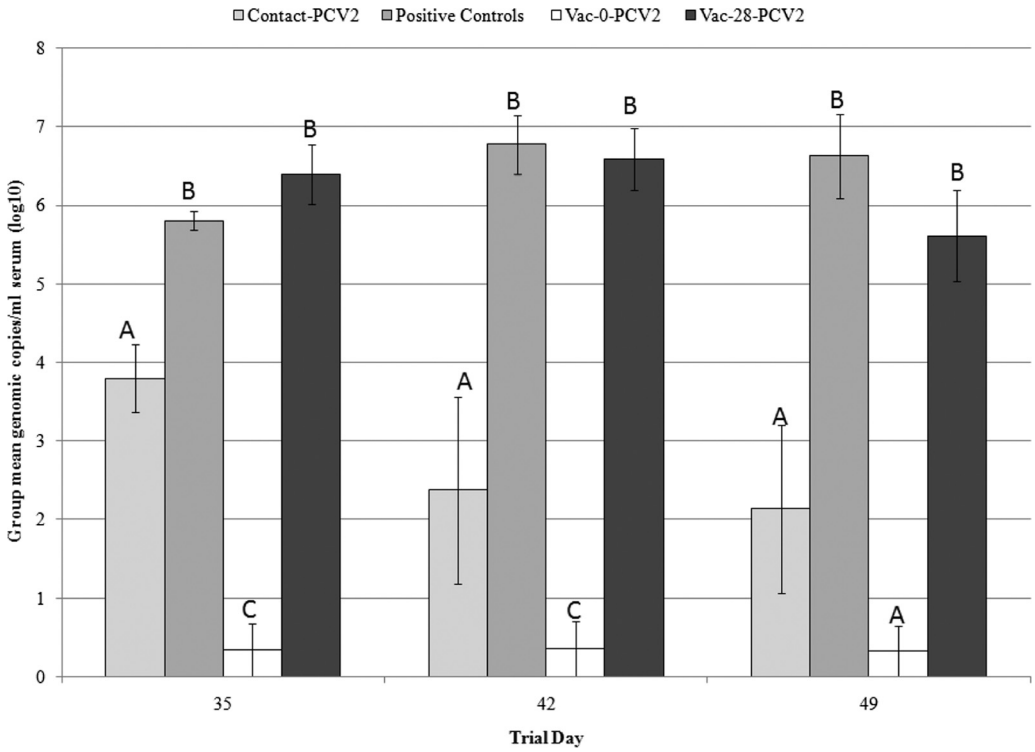

FIG. 2. Log-transformed group means for numbers of PCV2 DNA copies per ml serum. Pigs in groups Vac-0 and Vac-0–PCV2 were PCV1-2 vaccinated at trial day 0. Pigs in group Vac-28–PCV2 were PCV1-2 vaccinated at trial day 28. Contact-PCV2 pigs were nonvaccinated but commingled with Vac-0–PCV2 pigs. PCV2-PRRSV-PPV challenge was done at trial day 28. Different letters (A, B, C) on a given trial day indicate significant ($P < 0.05$) differences between groups.

other pathogens is also of concern. In many of the cases that we have investigated, vaccine failure is often determined to be due to actual failure to appropriately administer the vaccine to the pigs. All currently available commercial PCV2 vaccines are inactivated products. Many livestock producers and veterinarians prefer to use modified-live vaccines if they are available since they typically require only one dose with reduced costs. The objectives of this study were to investigate the safety and the efficacy of a live chimeric PCV2 vaccine based on subtype 2b in a PCV2b-PPV-PRRSV triple-coinfection model.

We have previously tested the efficacy of the PCV1-2a vaccine in experimental models using a single PCV2a challenge (10, 43), and we also investigated the efficacy of the live PCV1-2b vaccine using a single PCV2b challenge (4). Recently, the ability of the live chimeric PCV2 vaccine based on the 2b subtype to provide cross-protection against PCV2a and PCV2b has been reported (4). Moreover, the live PCV1-2a vaccine was recently tested and found to be efficacious in the PCV2b-PRRSV-PPV triple-challenge model (41). In this study we investigated the potential of pig-to-pig transmission of the live chimeric PCV1-2b vaccine between vaccinated and nonvaccinated naïve contact pigs and the safety of the live chimeric PCV1-2b in vaccinated pigs simultaneously infected with PRRSV and PPV, and we also investigated the efficacy of this subtype 2b-based vaccine in protecting pigs against PCV2b challenge in the PCV2-PRRSV-PPV triple-challenge model.

TABLE 4. Overall lymphoid lesion scores and prevalence of PCV2 antigen in lymphoid tissues[a]

Group	Overall lymphoid lesion score	No. of pigs with the following/total no. in group (%)		
		PCV2 antigen	Moderate lesions	Severe lesions
Negative controls	0.0 ± 0.0^{A}	0/8	0/8	0/8
Positive controls	$3.6 \pm 0.9^{B,C}$	8/8 (100)	4/8 (50)	1/8 (12.5)
Vac-0	0.2 ± 0.1^{A}	2/9 (22.2)	0/9	0/9
Vac-0–PCV2	0.2 ± 0.1^{A}	0/9	0/9	0/9
Contact-PCV2	$0.6 \pm 0.5^{A,B}$	2/3 (66.7)	0/3	0/3
Vac-28–PCV2	4.4 ± 0.9^{B}	8/9 (88.9)	5/9 (62.5)	2/9 (22.2)

[a] Data represent group mean ± standard error overall lymphoid lesion score and prevalence of PCV2 antigen in lymphoid tissues as determined by immunohistochemical stains at the time of necropsy (trial day 49, 21 days after PCV2b-PRRSV-PPV challenge). Different superscripts within columns (A, B, C) indicate significant ($P < 0.05$) differences between groups.

On the basis of the presence of severe clinical disease (1/8 positive-control pigs) consistent with systemic PCVAD and microscopic confirmation (1/8 positive-control pigs and 3/9 Vac-28–PCV2 pigs), the challenge used in the study was successful, and the results were similar to what has been previously described in other PCV2 coinfection studies (2, 3, 16, 20, 28, 35, 39). PCV2 replication was enhanced in nonvaccinated pigs, as evidenced by significantly ($P < 0.05$) more severe macroscopic and microscopic lesions and significantly ($P < 0.05$) higher serum and tissue PCV2 loads in the positive-control pigs than Vac-0–PCV2 pigs. For the PPV inoculum, we used a pig-passaged lung homogenate. It is possible that other unknown pathogens were propagated and present in this ho-

mogenate since it was not tested for ubiquitous viruses such as TTV, swine lymphotropic herpesvirus, and pestivirus. However, even if this were the case, it should not have affected the study outcome, as all challenged pigs received the same inoculum.

Our research group has previously studied the shedding and infection dynamics of PCV2b in animals naturally and experimentally infected (37, 38). With the knowledge that we gained from these studies, the question of whether a live-attenuated chimeric virus could also be transmitted between animals developed. Potential transmission of the live chimeric PCV2 vaccine was investigated by commingling vaccinated pigs with nonvaccinated contact pigs. Specifically, we placed nonvaccinated pigs (contact-PCV2) in the same room with pigs that had been vaccinated with the live PCV1-2b vaccine (Vac-0–PCV2) 3 days previously. Due to funding and space restraints, we were able to utilize only three nonvaccinated contact pigs; nevertheless, we found that the contact-PCV2 pigs became positive for PCV1-2 DNA by trial day 28, and anti-PCV2 antibodies appeared in these pigs immediately after PCV2 challenge and at least 1 week prior to any recognizable anti-PCV2 antibody development in the unvaccinated positive controls, suggesting that exposure of the contact pigs to vaccinated ones was sufficient to transmit PCV1-2b from pig to pig and to elicit an anamnestic immune response. Interestingly, the contact-PCV2 pigs appeared to be protected against PCV2-PRRSV-PPV challenge, as they developed only mild microscopic PCV2-associated lung and lymphoid lesions which did not differ from the lesions in the Vac-0–PCV2 pigs. This finding indicates that although the PCV1-2b vaccine is shed and transmitted from pig to pig, it is able to induce protective immunity in nonvaccinated pigs. To further evaluate the degree and magnitude of horizontal spread of the live PCV1-2b, which is an important aspect and finding, additional experiments involving more contact pigs should be conducted in the near future.

Vaccine safety was evaluated by determining the effect of concurrent infection of pigs with the live chimeric PCV2 vaccine and other swine pathogens. For this objective, pigs were vaccinated with PCV1-2b and concurrently infected with two viral pathogens (PRRSV and PPV) that have been documented to enhance PCV2 replication and associated PCVADs in pigs. PRRSV infection of growing pigs typically results in respiratory disease, fever, and reduced weight gain (26). PPV infection is normally not associated with any clinical signs in growing pigs (2, 5). Two different time points for the coinfections were evaluated. One group was vaccinated with the chimeric PCV1-2b vaccine virus 4 weeks prior to PCV2-PRRSV-PPV challenge (Vac-0–PCV2), whereas the other group was vaccinated with the PCV1-2b vaccine virus and subsequently inoculated with PCV2, PRRSV, and PPV on the same day (Vac-28–PCV2). Interestingly, the amount of PCV1-2b vaccine virus DNA among the three vaccinated groups (Vac-0, Vac-0–PCV2, and Vac-28–PCV2) was not different at any of the time points investigated (Table 3), indicating that concurrent infection with PRRSV and PPV did not enhance the replication of the vaccine virus PCV1-2b, even though numerous previous studies clearly documented that coinfection of PRRSV and PPV with PCV2 enhanced wild-type PCV2 replication (2, 3, 16, 20, 28, 29, 39). The results suggest that the chimeric PCV2 vaccine, when used as a live-attenuated vaccine, is expected to be safe in pigs coinfected by PRRSV and PPV. To better evaluate the precise interactions between the different pathogens and the timeline of disease progression, future studies should include higher numbers and more groups of pigs, including pigs infected 1 to 2 days before or after vaccination.

Vaccine efficacy was determined by vaccinating pigs 28 days prior to triple challenge using PCV2b, PRRSV, and PPV. When Vac-0–PCV2 pigs were compared to nonvaccinated positive-control pigs, there were significantly ($P < 0.05$) fewer severe microscopic lesions in the lung and lymphoid tissues in the vaccinated pigs. Moreover, no clinical disease or PCVAD-like lesions were observed in the Vac-0–PCV2 pigs. In addition, the incidence of PCV2 viremia after challenge and the PCV2 antigen load in tissues were significantly ($P < 0.05$) reduced in vaccinated animals. A protective effect of vaccination was not observed when the PCV1-2b vaccine was administered at the same time as PCV2 infection (Vac-28–PCV2); however, this was not unexpected, as successful vaccination with modified live vaccines requires time for the development of an active and protective immune response. Besides the insufficient vaccination-challenge interval (same day), it is also possible that the acute-phase responses elicited by the different pathogens were limiting the replication of the live chimeric PCV2 vaccine. The main reason for including this group was to confirm potential upregulation of the chimeric PCV2 vaccine by simultaneous infection with PPV and PRRSV.

In this study, vaccination was done by using two routes, the intramuscular and the intranasal routes. This approach was mainly done to ensure that all pigs received equal amounts of the vaccine at the same point in time and to ensure that both the mucosal and the systemic immune systems were activated. This approach is not likely to be practical for use in the field. Further investigations using a route that can be used under field conditions need to be conducted.

In summary, under the conditions of this study, we demonstrated that a live chimeric PCV2 vaccine based upon the PCV2b subtype was not upregulated by concurrent PPV and PRRSV infection and that it did induce protective immunity in a growing-pig coinfection model. To our knowledge, this is the first efficacy study of the live PCV1-2b vaccine virus in pigs coinfected with three common swine viral pathogens. The use of the chimeric PCV1-2b vaccine virus to cost-effectively and safely vaccinate large populations of pigs via the oral vaccination route appears to hold great potential.

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REFERENCES

1. **Allan, G. M., and J. A. Ellis.** 2000. Porcine circoviruses: a review. J. Vet. Diagn. Invest. **12:**3–14.
2. **Allan, G. M., et al.** 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. **121:**1–11.
3. **Allan, G. M., et al.** 2000. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. Arch. Virol. **145:**2421–2429.
4. **Beach, N. M., S. Ramamoorthy, T. Opriessnig, S. Q. Wu, and X. J. Meng.** 2010. Novel chimeric porcine circovirus (PCV) with the capsid gene of the

emerging PCV2b subtype cloned in the genomic backbone of the nonpathogenic PCV1 is attenuated in vivo and induces protective and cross-protective immunity against PCV2b and PCV2a subtypes in pigs. Vaccine **29:**221–232.
5. **Brown, T. T., Jr., P. S. Paul, and W. L. Mengeling.** 1980. Response of conventionally raised weanling pigs to experimental infection with a virulent strain of porcine parvovirus. Am. J. Vet. Res. **41:**1221–1224.
6. **Cheung, A. K., et al.** 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch. Virol. **152:**1035–1044.
7. **Ellis, J. A., G. Allan, and S. Krakowka.** 2008. Effect of coinfection with genogroup 1 porcine torque teno virus on porcine circovirus type 2-associated postweaning multisystemic wasting syndrome in gnotobiotic pigs. Am. J. Vet. Res. **69:**1608–1614.
8. **Fachinger, V., R. Bischoff, S. B. Jedidia, A. Saalmüller, and K. Elbers.** 2008. The effect of vaccination against porcine circovirus type 2 in pigs suffering from porcine respiratory disease complex. Vaccine **26:**1488–1499.
9. **Fenaux, M., et al.** 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J. Virol. **76:**541–551.
10. **Fenaux, M., T. Opriessnig, P. G. Halbur, F. Elvinger, and X. J. Meng.** 2004. A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J. Virol. **78:**6297–6303.
11. **Fenaux, M., T. Opriessnig, P. G. Halbur, and X. J. Meng.** 2003. Immunogenicity and pathogenicity of chimeric infectious DNA clones of pathogenic porcine circovirus type 2 (PCV2) and nonpathogenic PCV1 in weanling pigs. J. Virol. **77:**11232–11243.
12. **Fort, M., et al.** 2008. Porcine circovirus type 2 (PCV2) vaccination of conventional pigs prevents viremia against PCV2 isolates of different genotypes and geographic origins. Vaccine **26:**1063–1071.
13. **Gagnon, C. A., N. Music, G. Fontaine, D. Tremblay, and J. Harel.** 2010. Emergence of a new type of porcine circovirus in swine (PCV): a type 1 and type 2 PCV recombinant. Vet. Microbiol. **144:**18–23.
14. **Gillespie, J., et al.** 2008. A genetically engineered chimeric vaccine against porcine circovirus type 2 (PCV2) is genetically stable in vitro and in vivo. Vaccine **26:**4231–4236.
15. **Halbur, P. G., et al.** 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet. Pathol. **32:**648–660.
16. **Harms, P. A., et al.** 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. **38:**528–539.
17. **Horlen, K. P., et al.** 2008. A field evaluation of mortality rate and growth performance in pigs vaccinated against porcine circovirus type 2. J. Am. Vet. Med. Assoc. **232:**906–912.
18. **Huang, Y. W., B. A. Dryman, W. Li, and X. J. Meng.** 2009. Porcine DC-SIGN: molecular cloning, gene structure, tissue distribution and binding characteristics. Dev. Comp. Immunol. **33:**464–480.
19. **Kixmöller, M., et al.** 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. Vaccine **26:**3443–3451.
20. **Krakowka, S., et al.** 2000. Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine parvovirus. Vet. Pathol. **37:**254–263.
21. **Mankertz, A., J. Mankertz, K. Wolf, and H. J. Buhk.** 1998. Identification of a protein essential for replication of porcine circovirus. J. Gen. Virol. **79:**381–384.
22. **Mengeling, W. L.** 1979. Prenatal infection following maternal exposure to porcine parvovirus on either the seventh or fourteenth day of gestation. Can. J. Comp. Med. **43:**106–109.
23. **Mengeling, W. L., J. F. Ridpath, and A. C. Vorwald.** 1988. Size and antigenic comparisons among the structural proteins of selected autonomous parvoviruses. J. Gen. Virol. **69:**825–837.
24. **Nawagitgul, P., et al.** 2002. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. Clin. Diagn. Lab. Immunol. **9:**33–40.
25. **Nawagitgul, P., et al.** 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. **81:**2281–2287.
26. **Nodelijk, G.** 2002. Porcine reproductive and respiratory syndrome (PRRS) with special reference to clinical aspects and diagnosis. A review. Vet. Q. **24:**95–100.
27. **Opriessnig, T., et al.** 2006. Evidence of breed-dependent differences in susceptibility to porcine circovirus type-2-associated disease and lesions. Vet. Pathol. **43:**281–293.
28. **Opriessnig, T., et al.** 2004. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfected with type 2 porcine circovirus and porcine parvovirus. Vet. Microbiol. **98:**209–220.
29. **Opriessnig, T., et al.** 2008. Effect of porcine circovirus type 2 (PCV2) vaccination on porcine reproductive and respiratory syndrome virus (PRRSV) and PCV2 coinfection. Vet. Microbiol. **131:**103–114.
30. **Opriessnig, T., X. J. Meng, and P. G. Halbur.** 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. **19:**591–615.
31. **Opriessnig, T., A. R. Patterson, J. Elsener, X. J. Meng, and P. G. Halbur.** 2008. Influence of maternal antibodies on efficacy of porcine circovirus type 2 (PCV2) vaccination to protect pigs from experimental infection with PCV2. Clin. Vaccine Immunol. **15:**397–401.
32. **Opriessnig, T., A. R. Patterson, D. M. Madson, N. Pal, and P. G. Halbur.** 2009. Comparison of efficacy of commercial one dose and two dose PCV2 vaccines using a mixed PRRSV-PCV2-SIV clinical infection model 2-3-months post vaccination. Vaccine **27:**1002–1007.
33. **Opriessnig, T., et al.** 2010. Comparison of the effectiveness of passive (dam) versus active (piglet) immunization against porcine circovirus type 2 (PCV2) and impact of passively derived PCV2 vaccine-induced immunity on vaccination. Vet. Microbiol. **142:**177–183.
34. **Opriessnig, T., et al.** 2008. Differences in virulence among porcine circovirus type 2 isolates are unrelated to cluster type 2a or 2b and prior infection provides heterologous protection. J. Gen. Virol. **89:**2482–2491.
35. **Opriessnig, T., et al.** 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. Vet. Pathol. **41:**624–640.
36. **Opriessnig, T., et al.** 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet. Pathol. **40:**521–529.
37. **Patterson, A. R., D. M. Madson, P. G. Halbur, and T. Opriessnig.** 2011. Shedding and infection dynamics of porcine circovirus type 2 (PCV2) after natural exposure. Vet. Microbiol. **149:**225–229.
38. **Patterson, A. R., et al.** 2011. Shedding and infection dynamics of porcine circovirus type 2 (PCV2) after experimental infection. Vet. Microbiol. **149:**91–98.
39. **Rovira, A., et al.** 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J. Virol. **76:**3232–3239.
40. **Segalés, J., et al.** 2008. PCV-2 genotype definition and nomenclature. Vet. Rec. **162:**867–868.
41. **Shen, H. G., et al.** 2010. Comparison of commercial and experimental porcine circovirus type 2 (PCV2) vaccines using a triple challenge with PCV2, porcine reproductive and respiratory syndrome virus (PRRSV), and porcine parvovirus (PPV). Vaccine **28:**5960–5966.
42. **Sorden, S. D., P. A. Harms, P. Nawagitgul, D. Cavanaugh, and P. S. Paul.** 1999. Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J. Vet. Diagn. Invest. **11:**528–530.
43. **Thomas, P. J., T. Opriessnig, N. M. Juhan, X. J. Meng, and P. G. Halbur.** 2007. Planned exposure to porcine circovirus type 2 by serum injection is not effective at preventing porcine circovirus associated disease. J. Swine Health Prod. **15:**330–338.
44. **Tischer, I., H. Gelderblom, W. Vettermann, and M. A. Koch.** 1982. A very small porcine virus with circular single-stranded DNA. Nature **295:**64–66.